REMARKS

Claims 3, 4, 6-8, 11-28, and 32-46 are pending in the present application. Claims 6, 13, 32 and 45 have been amended. Upon entry of the present amendment, claims 3, 4, 6-8, 11-28 and 32-46 will remain pending.

I. The Claimed Invention Is Useful

Claims 3, 4, 6-8, 11-28, and 32-46 are rejected under 35 U.S.C. § 101 as allegedly failing to be supported by specific, substantial, or a well-established utility. Applicants traverse the rejection and respectfully request reconsideration thereof because the claims are supported by at least one specific, substantial, and credible utility.

The Revised Utility Examination Guidelines require a claimed invention to have a utility that is specific to the subject matter claimed (a "specific utility"). As recited in the claims, for example, the recombinant nucleic acid molecules can be used to induce an immune response against hepatitis C virus in a mammal and to treat a human who is infected with hepatitis C virus. Thus, there is no question that Applicants have asserted at least one **specific** utility and, in fact, have provided numerous specific utilities. Indeed, the specific utilities taught by Applicants are in stark contrast to the examples of the non-specific utilities (e.g., probes and chromosome markers without a specific DNA target) recited in the Revised Utility Examination Guidelines. Contrary to the assertions in the Office Action, Applicants are not required, under the Revised Utility Examination Guidelines, to set forth the function of the 5' untranslated region of hepatitis C virus. Thus, Applicants have complied with the specific utility requirement.

The Revised Utility Examination Guidelines also require a claimed invention to have a utility that defines a real-world use (a "substantial utility"). As set forth above, the recombinant nucleic acid molecules can be used to induce an immune response against hepatitis C virus in a mammal and to treat a human who is infected with hepatitis C virus. Thus, it is clear that the claimed invention has real-world uses. These real-world uses stand in stark contrast to the "throw away" uses (e.g., using transgenic mouse as snake food and using protein as an animal food supplement or shampoo) recited in the Revised Utility Examination Guidelines. The Office Action mistakenly

asserts that these are not substantial utilities and appears to require Applicants to set forth in the specification the mechanism of action and function of the 5' untranslated region of the hepatitis C virus (see, Office Action at pages 3-4). **No such requirement is mandated or even suggested in the Revised Utility Examination Guidelines.** Thus, there is no question that Applicants have asserted at least one substantial utility and, in fact, have provided numerous substantial utilities.

In addition to a specific and substantial utility, as Applicants have asserted, the Revised Utility Examination Guidelines require that such utility be credible (a "credible utility"). That is, whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided. Clearly, the numerous specific and substantial utilities asserted by Applicants are credible. Such assertions are credible unless "(A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based is inconsistent with the logic underlying the assertion." See, Revised Interim Utility Guidelines Training Materials. Further, PTO personnel are reminded that they must treat as true a statement of fact made by Applicants in relation to an asserted utility, unless countervailing evidence can be provided that shows that one of ordinary skill in the art would have a legitimate basis to doubt the credibility of such a statement. Significantly, no such countervailing evidence has been provided. If such evidence is available to the Examiner, Applicants request that the Examiner provide an affidavit pursuant to 37 C.F.R. §1.104(d)(2) containing evidence substantiating this position. Because Applicants have asserted numerous specific and substantial utilities that are credible, Applicants have complied with the credible utility requirement.

Thus, the claimed inventions clearly have a specific, substantial, and credible utility and, thus, are patentable subject matter. Accordingly, Applicants request that the rejection of claims 3, 4, 6-8, 11-28, and 32-46 under 35 U.S.C. § 101 and under 35 U.S.C. § 111 first paragraph be withdrawn.

II. The Claimed Invention Is Novel

Claims 3, 4, 6-8, 10-16, 45 and 46 are rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by U.S. Patent No. 6,297,048 (hereinafter, the "Jolly reference"). Applicants traverse the rejection and request reconsideration in view of the amended claims.

The Office Action asserts that the Jolly reference reports a recombinant retrovirus which expresses hepatitis C virus NS3/NS4, host cells comprising the vector, and pharmaceutical compositions comprising the nucleic acid molecule. Applicants are unable to locate, however, where the Jolly reference teaches that the nucleic acid molecules comprise a 5' untranslated region of hepatitis C virus, which is recited in each of amended claims 6, 13 and 45. Thus, the Jolly reference does not teach the claimed nucleic acid molecules, recombinant host cells, or pharmaceutical compositions. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 102(e) be withdrawn.

III. The Claimed Inventions Are Enabled

Claims 3, 4 and 6-8 are rejected under 35 U.S.C. § 112, first paragraph as allegedly failing to provide an enabling disclosure. The Office Action asserts that the claims are enabled for a recombinant nucleic acid molecule comprising a nucleotide sequence encoding hepatitis C virus NS3, NS4, or NS5 protein, operably linked to particular genetic elements. The Office Action, however, mistakenly asserts that the specification does not enable the same nucleic acid molecule operably linked to a 5' untranslated region of hepatitis C virus. Significantly, the Office Action provides absolutely no reasoning or evidence supporting such an erroneous conclusion. Indeed, no amount of undue experimentation is required to make or use such a nucleic acid molecule. Indeed, only routine technology is used to simply add the 5' untranslated region of hepatitis C virus to the nucleic acid molecule acknowledged as being enabled. Further, the nucleic acid molecule operably linked to a 5' untranslated region of hepatitis C virus can be used in the same manner as the nucleic acid molecule that is not operably linked to a 5' untranslated region of hepatitis C virus. In any event, the Office Action is devoid of any support for its position.

In view of the foregoing, there is no reason to believe that one skilled in the art would be required to perform undue experimentation to make and use the claimed invention as recited in claims 3, 4 and 6-8. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 112, first paragraph be withdrawn.

Claims 11-28 and 32-46 are rejected under 35 U.S.C. § 112, first paragraph as allegedly failing to provide an enabling disclosure. Applicants traverse the rejection and request reconsideration because one skilled in the art would be able to practice the claimed invention without being required to perform undue experimentation.

The Office Action asserts that it is "conventional" to make and use a nucleic acid molecule comprising a promoter, enhancer, and polyA which are operably linked to a nucleotide sequence encoding a protein. The Office Action, however, asserts that is not "conventional" to make such a nucleic acid molecule comprising a 5' untranslated region of hepatitis C virus. The Office Action further asserts that the prior art does not teach the function of the 5' untranslated region of hepatitis C virus or how to use an expression vector that comprised the 5' untranslated region of hepatitis C virus. Applicants submit that none of these reasons is at all relevant in an enablement rejection and only serves to point out the novelty of Applicants' claimed invention. The Office Action fails to point out what undue experimentation is required to make a nucleic acid molecule comprising a 5' untranslated region of hepatitis C virus. Indeed, only routine experimentation is required to make such genetic constructs. The Office Action also does not identify what undue experimentation must be carried out to use the claimed molecules. Indeed, a nucleic acid molecule comprising a 5' untranslated region of hepatitis C virus can be used in the same manner as a nucleic acid molecule that does not comprise a 5' untranslated region of hepatitis C virus. The Office Action provides no credible reasoning or evidence to support the enablement rejection.

The Office Action also asserts that the specification does not teach one skilled in the art how the 5' untranslated region of hepatitis C virus could be operably linked to the nucleotide sequence encoding a hepatitis C virus protein. Such procedures are, however, routine as evidence by Tokushige *et al.*, *Hepatology*, **1996**, *24*, 14-20, a copy of which is enclosed herewith (see, for

example, Figure 1A). Thus, no amount of undue experimentation is required to carry out the claimed inventions.

The Office Action also maintains that it would require undue experimentation for one skilled in the art to practice the claimed invention as it relates to prophylactic immunization of humans against HCV infection or therapeutic immunization against acquired infection. The reasoning provided in the Office Action is centered around two points -- first, the invention is allegedly drawn to "more than just an immune response" and, second, generation of immune responses in mice is not predictive of humans. Applicants traverse the rejection and request reconsideration because one skilled in the art would be able to practice the claimed invention without being required to perform undue experimentation.

Regarding the first reason, claims 17-28 recite methods of "inducing an immune response against hepatitis C virus in a human..." The claims do not recite methods of inducing a "protective" immune response. The Office Action has improperly tried to restrict Applicants' claimed invention by importing a limitation from the specification into the claims and then hold Applicants' claimed invention is not enabled. The enablement requirement of § 112 is satisfied so long as a disclosure contains sufficient information that persons of ordinary skill in the art having the disclosure before them would be able to make and use the invention. *In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988) (the legal standard for enablement under § 112 is whether one skilled in the art would be able to practice the invention without undue experimentation). In applying the enablement requirement, the "invention" that must be enabled is that defined by the claims. *Ex parte Erlich*, 3 U.S.P.Q.2d 1011 (Pat. Off. Bd. App. 1987). In *Erlich*, the Patent Office Board of Appeals was presented with a similar set of circumstances. The Examiner had rejected the claims there at issue (which related to processes for using monoclonal antibodies to isolate and purify human fibroblast interferon) because a screening assay step that was not required by the claims allegedly had not been enabled. Significantly, the Board reversed the Examiner's rejection, noting that the appealed claims did not require the assay step:

> The present disclosure as well as that of the parent application does enable one of ordinary skill in the art to practice the claimed invention. Thus, the claims on appeal are disclosed in the manner provided by 35 U.S.C. §112, first paragraph, ... and we reverse this rejection of the claims.

Erlich, 3 U.S.P.Q.2d at 1014. (emphasis in original). As in *Erlich*, Applicants have enabled practice of the claimed methods and compositions. As in *Erlich*, the Examiner's rejection is improper because it is directed to different methods. Indeed, "protective" is not recited in the claims. That some embodiments of Applicants' specification may recite that the methods provide "protective" immunity is of no moment in regard to the claimed invention. Indeed, any amount of immune response generated by the claimed methods would be beneficial to a human in whom the immune response is generated. In addition, any amount of immune response would be considered by a person skilled in the art to be a therapeutic amount. Indeed, Applicants are not claiming a method of "curing" a hepatitis C infection. In any event, an immune response generated in an individual would have both a therapeutic and prophylactic effect. Thus, Applicants have enabled the claimed invention.

The Office Action asserts that *Erlich* is not applicable because the issue is whether the claims are enabled for their intended use. *Erlich*, however, is clearly applicable in view of the fact that the Office Action is addressing enablement regarding a particular element -- a protective response or curing -- that is not recited in the claims. It is the **claimed invention** that must be enabled. In this regard, the Office Action has failed to present any credible reasoning or evidence rebutting Applicants' assertion that any amount of immune response generated by the claimed methods would be beneficial to a human in whom the immune response is generated. Thus, *Erlich* stands as controlling case law.

The Office Action also mistakenly maintains that it would require undue experimentation for one skilled in the art to practice the claimed invention in humans. The Office Action maintains that, despite the data regarding the humoral, cytotoxic, and anti-tumor effects in mice (*see*, Figures 2, 3 and 4 of the specification), the results in mice cannot be extrapolated to humans. The only reasoning provided in support of this assertion is that because mice cannot be infected with HCV, the response to an HCV vaccine in mice cannot be extrapolated to humans. Applicants recognize that mice do not outwardly express the disease state of a hepatitis C virus infection. Mice are, however, capable of eliciting an immune response to HCV, as is shown throughout Applicants' specification. Further, the title of Encke *et al.*, *J. Immunol.*, **1998**, *161*, 4917-4923 (hereinafter, the "Encke

reference") (*i.e.*, "Genetic Immunization Generates Cellular and Humoral Immune Responses Against the Nonstructural Proteins of the Hepatitis C Virus in a Murine Model") clearly teaches that mice are, indeed, an animal model for inducing immune responses.

In view of the data regarding the humoral, cytotoxic, and anti-tumor effects of the claimed compositions in mice, one skilled in the art would have no reason to doubt that an immune response would be generated in humans. Applicants remind the Examiner that the claims are not directed to using a mouse model to evaluate the HCV disease state in response to particular compounds. Rather, the claims are directed to methods of inducing an immune response. The Office Action provides no evidence that a human would fail to generate an immune response to the claimed compositions. Further, the Office Action provides no credible reasoning or evidence supporting the assertion that one skilled in the art would not expect an immune response to be generated in a human despite the fact that an immune response is generated in a mouse.

Referral in the Office Action to Houghton, *Current Topics in Microbiology and Immunology*, **1999**, pp327-339 (hereinafter, the "Houghton reference") does not support the position taken in the Office Action. The Houghton reference does not teach or suggest that results in mice cannot be extrapolated to humans. Indeed, the Houghton reference refers to studies with mice, guinea pigs, and chimpanzees. The notion that dosages may have to be adjusted from one animal to another in no way points out the lack of expected success in humans. Further, that efficacy studies are suggested to be carried out in primates, such as chimpanzees, is of no surprise in view of the FDA's requirements for safety and the like. Neither the PTO nor the patent laws, however, are charged with the duties of the FDA. Thus, the Houghton reference does not teach or suggest that the humoral, cytotoxic, and anti-tumor effects of the claimed compositions in mice would not also be expected to be generated in humans.

Further, referral in the Office Action to Chattergoon, *FASEB J.*, **1997**, *11*, 753-763 (hereinafter, the "Chattergoon reference") not only fails to support the positions taken in the Office Action but, in fact, supports Applicants' position. The Office Action asserts that the Chattergoon reference "clearly points out" that there is no evidence of success of using DNA vaccines in humans. In contrast, the Chattergoon reference states:

> The ability of plasmid DNA to induce immune responses after inoculation has been demonstrated in several animal models. Further, within the limits of these disease models, the immune responses elicited by DNA vaccines **have been shown to be protective**. (emphasis added)

(page 762, left column). The only evidence that the Chattergoon reference points out is that there is little evidence that the immune responses will be "completely" protective against any human pathogen. Indeed, as stated above, Applicants' claims neither recite nor require "complete" protective immunity. Rather, Applicants' claims are directed to inducing an immune response. In addition, Table 1 of the Chattergoon reference actually points out that the investigator "Ray" has identified HCV as a target of DNA vaccines by in which "mouse/rat" has been identified as the animal model. Thus, the Chattergoon reference does not support the position taken in the Office Action.

Finally, the statement in the Encke reference referred to in the Office Action also does not support the position taken therein. The Office Action appears to assert that the Encke reference teaches that mice are not predictive of humans. The sentence from the Encke reference referred to in the Office Action states:

> However, the clinical efficacy of DNA-based immunization in generating antiviral immune responses against HCV in humans remains to be established.

(page 4922-4923). Thus, the authors of the Encke reference refer to the "clinical efficacy" of the DNA vaccines, not whether the DNA vaccines will generate an immune response. That a complete protective immune response that is also clinically safe may indeed remain to be seen. As stated numerous times, however, Applicants' claims are not directed to curing an HCV infection but, rather, recite methods of "inducing an immune response."

In contrast to the assertions in the present Office Action, these references, as set forth above, **do not** show the unpredictability of the state of the art. The present Office Action fails to address any of the foregoing arguments regarding the art cited in the previous Office Action.

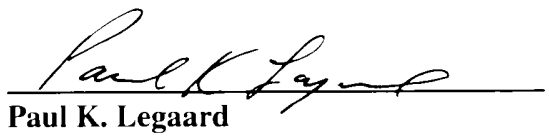
In view of the foregoing, there is no reason to believe that one skilled in the art would be required to perform undue experimentation in order to make and use the claimed invention to induce

an immune response in humans. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 112, first paragraph be withdrawn.

IV. Conclusion

In view of the foregoing, Applicants respectfully submit that the claims are in condition for allowance. An early notice of the same is earnestly solicited. The Examiner is invited to contact Applicants' undersigned representative at (215) 564-8906 if there are any questions regarding Applicants' claimed inventions. Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned **"Version with markings to show changes made."**

Respectfully submitted,



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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

Claims 6, 13, 32 and 45 have been amended as follows:

6. (Amended three times) A recombinant nucleic acid molecule comprising a nucleotide sequence encoding hepatitis C virus NS4 or NS5 protein wherein said nucleotide sequence is operably linked to a promoter, enhancer, polyadenylation sequence, and [optionally] 5' UTR of hepatitis C virus.

13. (Amended three times) A pharmaceutical composition comprising:

a) a recombinant nucleic acid molecule comprising a nucleotide sequence encoding hepatitis C virus NS4 or NS5 protein, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells; and

b) a pharmaceutically acceptable carrier or diluent;

wherein said regulatory elements functional in human cells comprise a promoter, enhancer, polyadenylation sequence, and [optionally] 5' UTR of hepatitis C virus.

32. (Amended three times) A method of treating a human who is infected with hepatitis C virus comprising administering to said human a pharmaceutical composition in an amount effective to induce a therapeutic immune response against hepatitis C virus, wherein said composition comprises a recombinant nucleic acid molecule comprising a nucleotide sequence encoding a hepatitis C virus nonstructural protein, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells, and a pharmaceutically acceptable carrier or diluent, wherein said regulatory elements functional in human cells comprise a promoter, enhancer, polyadenylation sequence, and [optionally] 5' UTR of hepatitis C virus.

45. (Amended) A pharmaceutical composition comprising:

a) a recombinant nucleic acid molecule comprising a nucleotide sequence encoding a hepatitis C virus nonstructural protein, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells;

b) a pharmaceutically acceptable carrier or diluent; and

c) a facilitator;

wherein said regulatory elements functional in human cells comprise a promoter, enhancer, polyadenylation sequence, and [optionally] 5' UTR of hepatitis C virus.

Expression and Immune Response to Hepatitis C Virus Core DNA–Based Vaccine Constructs

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Hepatitis C virus (HCV) is a major worldwide cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The development of vaccines against HCV have been complicated by the high variability of the envelope region, and it is likely that the cellular immune responses to viral structural proteins may be important for eradicating persistent viral infection. Recently, it was reported that the injection into muscle cells of plasmids encoding viral genes resulted in the generation of strong cellular immune responses. We constructed vectors that express the highly conserved HCV core gene. In this regard, the pHCV 2-2 construct contained the entire HCV core region and pHCV 4-2 contained both the 5′ noncoding region and the core gene. Cellular expression of HCV core protein was assessed following transfection into human and murine cell lines, and higher intracellular levels of the 21-kd core protein were observed with pHCV 2-2. These HCV core DNA constructs were used to immunize BALB/c mice and produced low-level anti-HCV core humoral immune responses. To assess cytotoxic T-lymphocyte (CTL) activity generated *in vivo*, a cloned syngeneic SP_2/O myeloma cell line constitutively expressing HCV core protein was established and inoculated into BALB/c mice to produce growth of plasmacytomas. Strong CTL activity was generated because the tumor size and weight in pHCV 2-2–immunized mice were remarkably reduced compared with mice injected with mock DNA. Spontaneous CTL activity was also exhibited by splenocytes in an *in vitro* cytotoxicity assay. These investigations demonstrate that plasmid constructs expressing HCV core protein generate strong CTL activity, as assessed both *in vivo* and *in vitro*, and are promising candidates as antiviral agents. (HEPATOLOGY 1996;24:14-20.)

Hepatitis C virus (HCV) is a positive-strand RNA virus with a linear genome of about 9,500 bases. Different isolates show considerable nucleotide sequence diversity, leading to the subdivision of HCV genomes in at least six genotypes.[1] In all genotypes, the viral RNA contains a large, open reading frame that encodes a polyprotein precursor of 3010 to 3033 amino acids.[2-6] This precursor is cleaved by cellular and viral proteinases to give rise to the core, envelope (E1, E2), and nonstructural proteins (NS2-NS5).[7-9] The coding sequence of the RNA genome is preceded by a 5′ noncoding region of 324 to 341 nucleotides,[10,11] which is highly conserved among all strains of HCV. This noncoding region forms an extensive and stable secondary structure,[12] and serves as an internal ribosomal entry site—essential for efficient cap-independent viral translation—and probably is also necessary for HCV replication.[13-15]

HCV is a major causative agent of posttransfusion hepatitis. More than 50% of acutely infected individuals progress to a chronic carrier state that frequently results in cirrhosis.[16] In addition, HCV infection is an independent risk factor for the development of hepatocellular carcinoma, as shown by the prevalence of anti-HCV antibodies.[17-20] Currently, there is no universal, highly effective therapy of chronic HCV infection. In several studies, it has been shown that the response rate of chronic HCV infection to interferon alfa treatment was as low as 15 to 25%.[21] Other antiviral nucleoside analogs have been shown to be only marginally effective[22] and may be associated with considerable side effects. Therefore, the development of alternative approaches is of major clinical importance.

There is a need to develop new antiviral agents in an attempt to eradicate persistent HCV infection from the liver. In this regard, direct injection of DNA into animals is a novel and promising method for delivering specific antigens for immunization.[15,23-26] This approach has been successfully used to generate protective immunity against influenza virus in mice and chickens, against bovine herpes virus 1 in mice and cattle, and against rabies virus in mice.[27-31] In most cases, strong, yet highly variable, antibody and cytotoxic T-lymphocyte (CTL) responses were associated with control of infection. Indeed, the potential to generate long-lasting memory CTL without using a live vector makes this approach particularly attractive compared with those involving killed-virus vaccines and subunit vaccines. This direct DNA-based genetherapeutic approach has shown great utility for generating a CTL response that not only protects against acute infection but also may have benefits in eradicating persistent viral infection,[24,26,28-38] of which HCV is an important human prototype. Because the HCV core gene is highly conserved among the various genotypes, we explored its use in a polynucleotide-based vaccine strategy to generate a CTL response in the host.

MATERIALS AND METHODS

Plasmids and Cell Lines. A complementary DNA encoding the HCV core gene was isolated from an anti-HCV–positive individual as described by Wakita et al.[14] This complementary DNA fragment was inserted into a plasmid expression vector containing a rous sarcoma virus enhancer element and driven by a cytomegalovirus promoter. As shown in Fig. 1A, one construct, designated pHCV 2-2, contains the entire HCV core gene, and the other, pHCV 4-2, contains the HCV core coding, as well as the 5′ noncoding region. The con-

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Abbreviations: HCV, hepatitis C virus; CTL, cytotoxic T lymphocyte.

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Presented, in part, at the 1995 Annual Meeting of the American Association for the Study of Liver Diseases and appeared in abstract form (HEPATOLOGY 1995;20:220A).

Supported by Grants CA-35711 and AA-02169 from the National Institutes of Health and the Tan Yan Kee Foundation. Dr. Moradpour is the recipient of a fellowship from the Swiss National Science Foundation.

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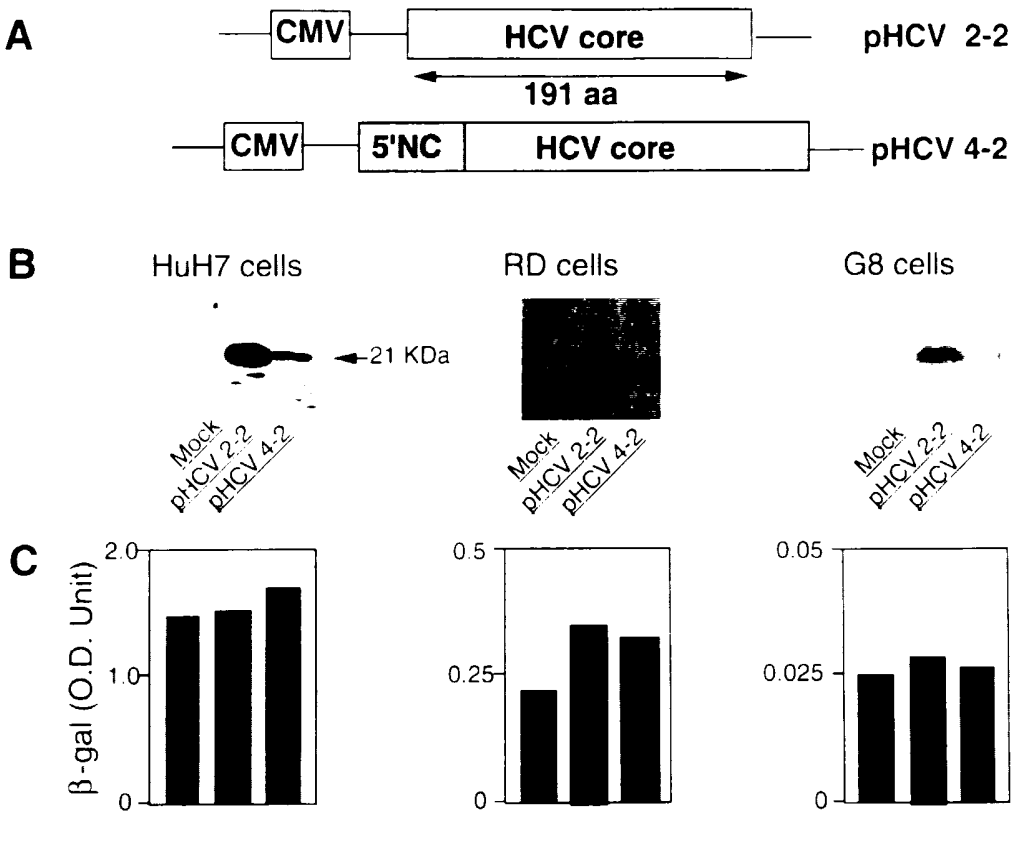

FIG. 1. Intracellular expression of the 21-kd HCV core protein. (A) Cartoon of the HCV core expression vector pHCV 2-2 that contains the entire HCV core gene, and pHCV 4-2 that has both the 5′ noncoding region and core coding region. (B) Western blot analysis demonstrates HCV core gene expression in HuH-7 human hepatocellular carcinoma cells and human (RD) and mouse (G8) muscle cell lines. (C) Transfection efficiency as measured by expression of β-galactosidase after cotransfection with HCV core DNA vaccine constructs at a 4:1 ratio of pHCV 2-2 or 4-2 to pSV-β gal.

structs were grown in DH5α cells, and plasmid DNA was purified by CsCl gradient ultracentrifugation as described.[35]

To assess intracellular levels of HCV core protein following transient transfection of plasmid constructs, HuH-7 cells, a human hepatoma cell line, RD cells, a human rhabdomyosarcoma cell line, and G8 cells, a mouse myoblast cell line, were employed. The SP_2/O syngeneic BALB/c mouse myeloma-derived cell line was used to generate target cells to measure CTL activity both *in vivo* and *in vitro*.[39] All cell lines were obtained from the American Tissue Culture Collection (Rockville, MD).

In Vitro *Studies*. The two HCV core DNA constructs were transfected into HuH-7, RD, and G8 cells by the calcium phosphate precipitation method.[40] Two days after transfection, cells were analyzed for 21-kd core protein expression by Western blot analysis. β-Galactosidase assays were performed following a standard protocol (Promega protocols and applications guide, 2nd ed., 1991, Promega Corp., Madison, WI), which were used to normalize Western blots for transfection efficiency. In brief, cell lysates were prepared in RIPA buffer (0.15 mol/L NaCl, 1% NP-40, 50 mmol/L Tris, 0.5% deoxycholate, and 1% sodium dodecyl sulfate), separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrotransferred onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking with 3% nonfat dry milk, membranes were incubated with a previously developed anti-HCV core monoclonal antibody, designated C7-50A,[41] followed by detection with a ^{125}I-labeled goat anti-mouse polyclonal antibody (Dupont, New England Nuclear, Billerica, MA). In addition, immunofluorescence staining was performed to determine the cellular localization of the HCV core protein as described previously.[41]

Plasmid DNA Immunization. Pathogen-free BALB/c female mice, aged 6 to 8 weeks, were purchased from Charles River Breeding Laboratory (Boston, MA) and used for all *in vivo* studies. To enhance muscle cell uptake of plasmid DNA, the quadriceps were injected first, at multiple sites with a total of 100 μL of 0.25% bupivacaine. Twenty-four hours after bupivacaine administration, 100 μg of plasmid DNA was injected into the same region at multiple sites. Thereafter, plasmid DNA constructs were injected every 2 weeks for a total of four intramuscular immunizations. Immunizations with recombinant HCV core-glutathione S-transferase fusion protein (1 μg)[42] was performed subcutaneously in complete Freund's adjuvant. BALB/c mice were inoculated every 2 weeks for a total of four immunizations.

Antibody Assays for HCV Core Antigen. To measure HCV core antibodies in serum of immunized mice, the following assay was developed and employed. In brief, a HCV core-GST fusion protein (0.5 μg per well) was used to coat microtiter plates (Falcon, Microtest IIIM Flexible Assay Plate, Pittsburgh, PA). Plates were incubated overnight at 4°C. After blocking with 3% bovine serum albumin in phosphate-buffered saline for 2 hours at 20°C, mouse serum diluted 100-fold was added to the plates and incubated at 20°C for an additional 2 hours. After washing with phosphate-buffered saline containing 0.05% Tween, 1×10^5 cpm of ^{125}I-labeled goat anti-mouse immunoglobulins G and M was added. Following a 1-hour incubation, plates were washed, and radioactivity bound to the plate was determined in a gamma well counter.

Cytotoxic T-Lymphocyte Activity. To assess CTL activity in BALB/c mice, a clonal syngeneic SP_2/O myeloma target cell line was established by stable transfection with a HCV core expression construct; this vector was driven by the elongation 1-α promoter.[43] Stable transfectants were selected in G418-containing medium. After cloning by limiting dilution, several SP_2/O cell lines expressing the HCV core protein were established. The presence of HCV core protein was demonstrated by immunofluorescent staining and by using the C7-50 monoclonal antibody. One week after the final immunization with plasmid DNA, 1×10^7 SP_2/O cells expressing the HCV core protein or native SP_2/O cells were inoculated intraperitoneally and subcutaneously into the right and left flank of the mouse. The tumor size and weight, as well as animal survival, were evaluated as an *in vivo* index of CTL activity generated by immunization with the various plasmid DNA constructs.

For *in vitro* studies, CTL activity was measured 1 week following SP_2/O myeloma cell inoculation. Mice were killed and a suspension of spleen cells was prepared from each animal. It is important to emphasize that splenocytes were not prestimulated with HCV core peptides or cells expressing HCV core peptides. Thus, this assay assesses spontaneous CTL activity present in the spleen of immunized mice. Native SP_2/O or SP_2/O cells expressing HCV core antigen were labeled with 100 μCi of ^{51}Cr (Amersham, Arlington Heights, IL) for 2 hours and washed three times with phosphate-buffered saline. CTL activity was determined in a standard 4-hour ^{51}Cr re-

lease assay using U-bottom 96-well plates containing $1 \cdot 10^4$ target cells per well. The percent specific release of ^{51}Cr-labeled target cells was calculated using the following formula: (experimental release − spontaneous release)/(total release − spontaneous release) · 100. Maximum ^{51}Cr release was determined by lysis of target cells with 5% Triton X-100. All assays were performed in triplicate.

Statistical Analysis. To compare differences between two groups, a Student's *t* test was used. *P* values < .05 were considered significant.

RESULTS

Intracellular expression of the 21-kd core protein following transfection with the pHCV 2-2 and pHCV 4-2 constructs was evaluated in HuH-7, RD, and G8 cells by Western blot analysis, as shown in Fig. 1B. Transfection efficiency was assessed by cotransfection with a β-galactosidase–expressing plasmid (Fig. 1C). It was surprising that pHCV 2-2 (Fig. 1B), which contains only the core gene, consistently gave higher intracellular levels of the 21-kd core protein as expressed in HuH-7, RD, and G8 cells compared with pHCV 4-2. There was only a minor difference in transfection efficiency between mock DNA and the two HCV-DNA constructs within the same cell line. Transfection efficiency was highest in HuH-7 cells, followed by the human (RD) and mouse (G8) muscle cell–derived cell lines. As shown in Fig. 2, core protein was found localized to the cytoplasm in cells transfected with the pHCV 2-2 construct. There was no secretion of HCV core protein into the culture supernatant as measured by a two-site monoclonal antibody–based immunoassay.[41]

The anti-HCV core humoral immune response to the various DNA constructs was assessed by measurement of antibodies in serum of mice following four intramuscular immunizations of 100 μg plasmid DNA. As shown in Fig. 3, pHCV 2-2–immunized mice demonstrated a 40% serocoversion rate, whereas only 20% of the mice immunized with pHCV 4-2 developed anti-HCV core antibodies. As a positive control, we compared the anti-HCV core antibody response in mice (n = 5) receiving four injections of recombinant HCV core-GST fusion protein (5 μg), and found that all animals seroconverted and that the anti-HCV core titers were considerably higher than in mice immunized with HCV core DNA constructs (data not shown).

To assess CTL activity *in vivo*, a mouse tumor model was established. As shown in Fig. 4A and B, syngeneic BALB/c-derived SP_2/O myeloma cells were stably transfected with a plasmid construct expressing the HCV core, as well as part of the envelope genes under the control of the elongation factor 1-α promoter. Such cells will express and present endogenously processed HCV core peptides in the context of major histocompatibility complex class I molecules on the cell surface to the immune system. A Western blot of the clonal SP_2/O cell line constitutively expressing the 21-kd HCV core protein is shown in Fig. 4B. Mice injected with this cell line will rapidly develop large plasmacytoma tumors. To maximally "challenge" the level of CTL activity, mice were inoculated with a large tumor (1×10^7 SP_2/O myeloma cells) burden into multiple sites, as illustrated in Fig. 4C. The experimental design involved four inoculations of either 100 μg mock DNA, pHCV 2-2, or pHCV 4-2 every 2 weeks, followed by a tumor cell challenge 1 week after the fourth and last injection. In this model system, if CTL activity is generated, there will be inhibition of tumor growth and increased animal survival rate.[44] Table 1 illustrates the tumor size 1 and 2 weeks after inoculation of 1×10^7 SP_2 O HCV core protein expressing myeloma cells in nonvaccinated mice compared with animals receiving the plasmid DNA (mock), pHCV 2-2, and pHCV 4-2 constructs. At 1 week, all mock DNA-immunized mice developed plasmacytomas at all injection sites. In the pHCV 2-2– or pHCV 4-2–inoculated mice, tumors were found at only 40% and 65% of the injection sites, respectively. Two weeks after challenge with this large SP_2 O myeloma cell burden, the mean tumor size was significantly reduced only in the pHCV 2-2–vaccinated mice. Table 2 demonstrates that the protective effects were specific, because native SP_2 O cells (without HCV core peptide expression) grew rapidly and equally well in all vaccinated mice.

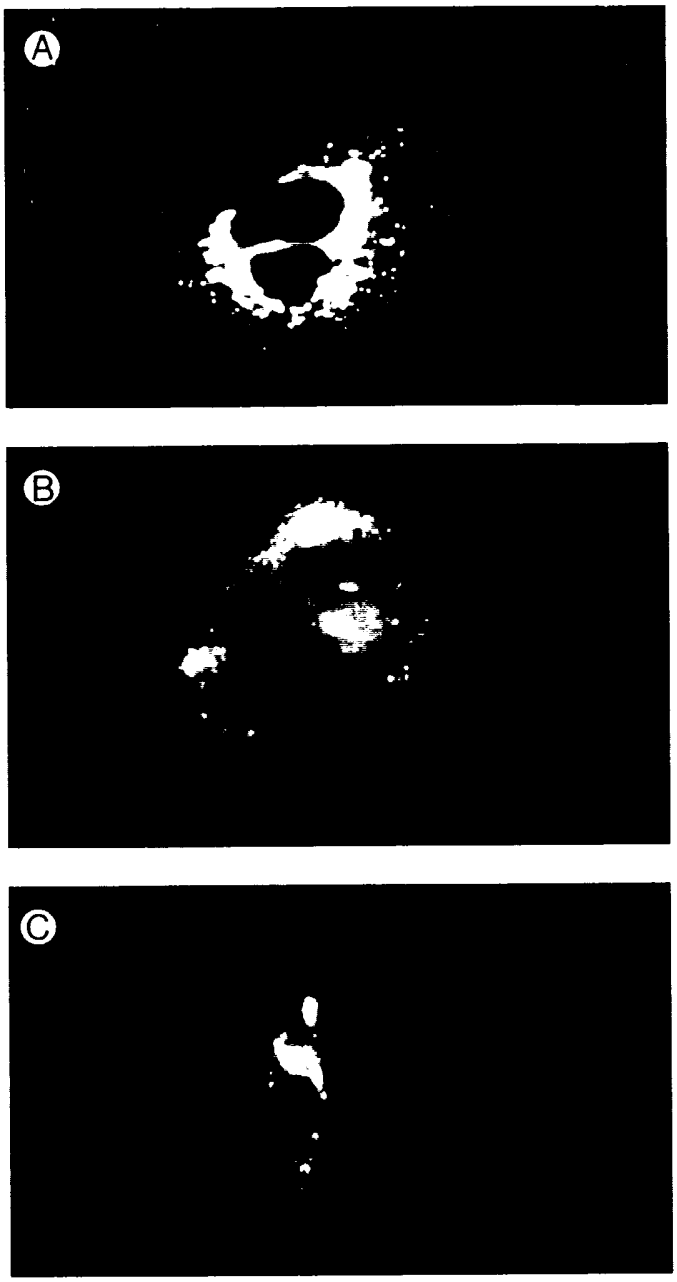

FIG. 2. Immunofluorescent studies of HCV core protein expression as detected by the C7-50 monoclonal antibody. Note the intense cytoplasmic staining of HCV core protein in (A) HuH-7 cells, (B) RD cells, and (C) G8 cells transiently transfected with the pHCV 2-2 construct.

Further support for generation of CTL activity is illustrated by the data presented in Fig. 5 with respect to overall tumor burden (weight) produced at the subcutaneous and intraperitoneal injection sites. Tumor weight was assessed 16 to 18 days after injection of 1×10^7 SP_2 O–HCV core myeloma cells. There was a significant reduction in tumor burden in animals vaccinated with pHCV 2-2. Finally, Fig. 6A shows representative examples of tumors in mice 2 weeks after inoculation with 1×10^7 SP_2 O–HCV core expressing cells. In Fig. 6A, mice immunized with mock DNA (right) are

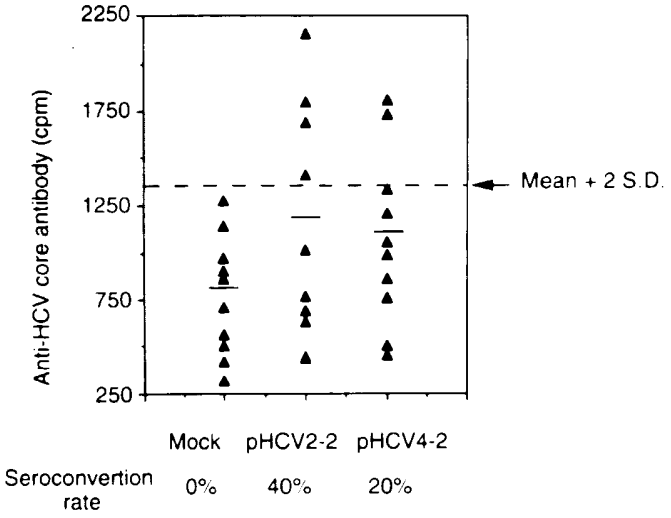

FIG. 3. Humoral immune response following four inoculations with 100 μg of either mock DNA construct or a pHCV 2-2 or pHCV 4-2, as measured by anti-HCV core binding activity in serum. Serum from 10 mice in each group was tested, and a positive result was defined as a value greater than the mean ± 2SD of the mock DNA–immunized control group.

TABLE 1. Tumor Size After Challenge With SP_2/O Cells Expressing HCV Core Antigen in Nonimmunized (normal) or Mice Receiving Intramuscular Injections of Mock DNA, pHCV 2-2, and pHCV 4-2 Constructs

DNA	Tumor Size: Not Detectable	5 mm	10 mm	20 mm	20 mm	mean ± SD
Normal	0	4	5	1	–	6.70 ± 3.75*†
(n = 5)	0	0	0	4	6	22.50 ± 7.49*
Mock	0	5	10	5	–	7.45 ± 4.18*‡
(n = 10)	0	0	0	5	15	22.50 ± 4.55*
pHCV 2-2	12	5	3	0	–	1.58 ± 2.30
(n = 10)	3	1	5	11	0	10.60 ± 6.63
pHCV 4-2	7	7	6	0	–	3.55 ± 3.12§
(n = 10)	0	0	1	10	7	19.33 ± 7.21
r-HCV	0	2	3	0	–	7.33 ± 2.36*†
(n = 3)	0	0	0	4	2	18.33 ± 7.21

NOTE. The first set of data represent findings at 1 week after tumor injection; the second set represent findings at 2 weeks after tumor injection.

Abbreviation: r-HCV, recombinant HCV core-GST fusion protein.

* $P < .0001$, pHCV 2-2 vs. other groups.

† $P < .05$, ‡ $P < .01$, § $P < .05$, ‖ $P < .01$, pHCV 4-2 vs. other groups.

compared with those vaccinated with pHCV 2-2 (left). Finally, as shown in Fig. 6B, survival rate was enhanced in mice immunized with pHCV 2-2 compared with pHCV 4-2 and mock DNA constructs.

To confirm the generation of CTL activity *in vivo*, experiments were performed with splenocytes *in vitro*. The assay measures spontaneous CTL activity because there was no prior stimulation of cells by HCV peptides. Figure 7 illustrates the CTL activity exhibited by splenocytes derived from mock DNA–, pHCV 2-2–, and pHCV 4-2–immunized mice at effector/target ratios of 50:1 and 100:1 against SP_2/O HCV core expressing cells compared with native SP_2/O target cells. It is noteworthy that splenocytes derived from pHCV 2-2 and pHCV 4-2–immunized mice specifically killed only the core antigen–expressing SP_2/O cells. We found that pHCV 2-2 was more effective in generating CTL activity than the pHCV 4-2 construct.

DISCUSSION

The high mutational rate of the HCV genome may be related to the establishment of persistent viral infection and subsequent disease chronicity.[16,45,46] The cellular immune

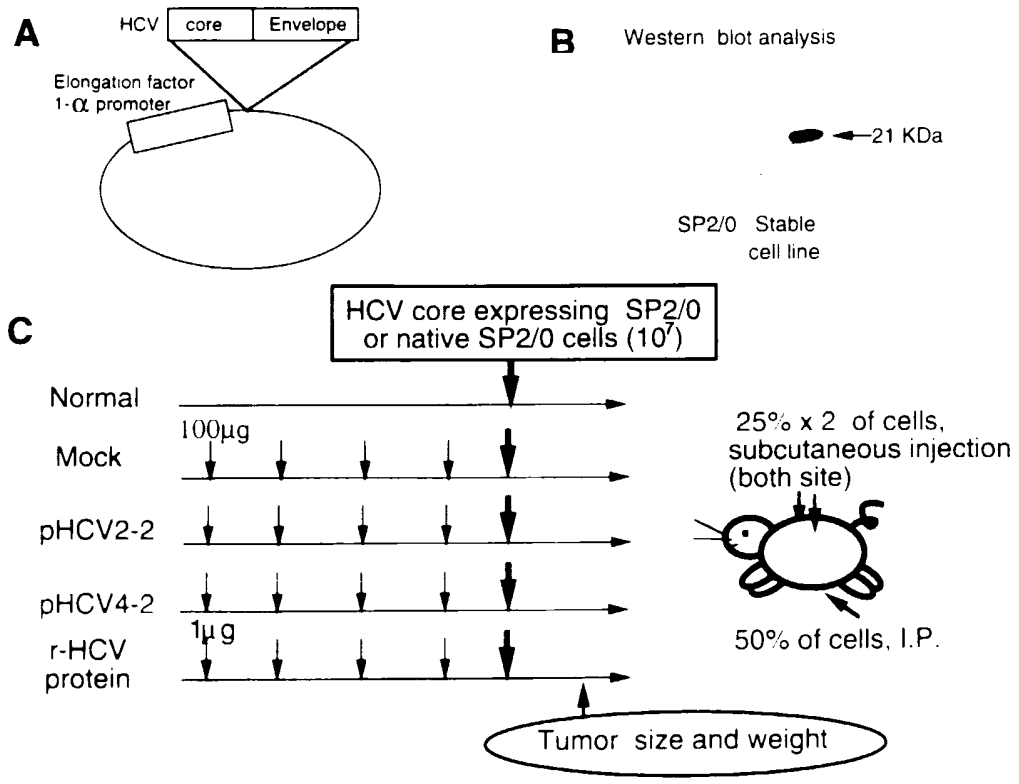

FIG. 4. Cartoon of the experimental design to assess CTL activity generated by the various vaccine constructs (n = 10). A, Expression vector used to establish stable HCV core–producing SP_2O myeloma cell lines. B, A clonal SP_2O cell line expressing the 21-kd core protein analyzed by Western blot analysis. C, Immunization schedule and production of plasmacytomas at various injection sites. r-HCV, recombinant HCV core-GST fusion protein.

TABLE 2. Tumor Size 1 Week Following Injection of Native SP_2O Cells

DNA	Tumor Size: Not Determined	5 mm	10 mm	20 mm	mean ± SD*
Mock (n = 5)	1	2	2	3	8.44 ± 4.73
pHCV 2-2 (n = 5)	1	2	3	4	8.80 ± 3.94
r-HCV (n = 3)	1	1	1	1	6.25 ± 3.79

Abbreviation: r-HCV, recombinant HCV core-GST fusion protein.
* There was no significant difference between the three groups.

events involved in liver damage and viral clearance during HCV infection have only partially been defined. In an attempt to examine a potential pathogenic role of liver-infiltrating lymphocytes in patients with chronic HCV infection, Koziel et al. examined the CTL response of such cells and demonstrated a HLA class I–restricted $CD8^+$ T-lymphocyte (CTL) response that was directed against both structural and nonstructural regions of HCV polypeptides.[47,48] Other investigators have also noted the existence of CTLs in peripheral blood mononuclear cell populations that recognize epitopes on core and the other viral-related proteins during chronic HCV infection.[49,50] Botarelli et al.[51] and Ferrari et al.[52] found HLA class II–restricted $CD4^+$ T-cell–mediated proliferative responses to several recombinant proteins derived from different regions of HCV in patients with chronic HCV infection. It is noteworthy that there was a correlation between T-cell responses to HCV core protein and a clinically benign course of the liver disease, as well as subsequent eradication of the virus. However, a similar study by Schupper et al.[53] showed that the proliferative response to HCV core protein did not predict a benign clinical course with respect to the severity of the liver disease.

We have recently studied peripheral blood mononuclear cell responses to a recombinant GST-HCV core fusion protein by evaluating the ability of such cells to produce interferon gamma, correlations were made to different clinical outcomes of HCV infection.[42] Individuals who had received interferon alfa treatment and went into clinical and virological emission had a higher response rate (75%, $P < .05$) to HCV core protein compared with those with ongoing hepatitis who failed therapy (31%). These clinical observations suggested to us that if one could augment the host cellular immune response to HCV core antigenic determinants, then it may be possible to enhance viral clearance during persistent infection.

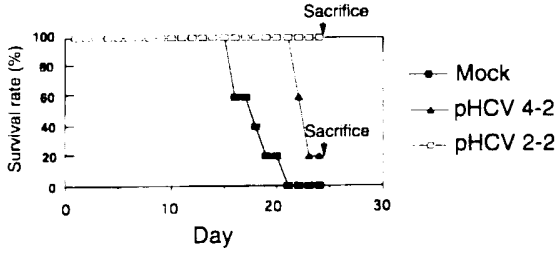

FIG. 6. Effect of immunization on the growth of SP_2O myeloma cells as an index of CTL activity. (A) Representative examples of tumor growth at 15 days in three mice immunized with pHCV 2-2 (left) compared with mock DNA (right). Note the large size of the tumors (arrows). (B) Survival rate of the vaccinated mice. Note that all mice (n = 10) immunized with pHCV 2-2 survived during the observation period, whereas all mock DNA–immunized controls (n = 10) died by 21 days.

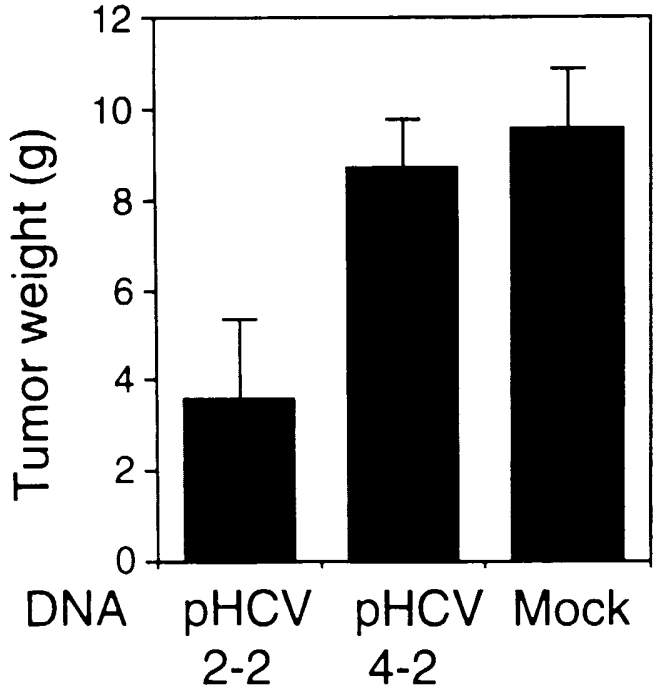

FIG. 7. Illustration of the tumor burden as an in vivo index of CTL activity generated 5 to 7 days after inoculation of 1×10^7 SP_2O HCV core expressing cells into BALB/c mice. Note the striking reduction of tumor weight in mice immunized with the pHCV 2-2 construct ($P < .001$ vs. mock DNA–immunized mice).

Relevant to this issue of polynucleotide-based induction of cellular and humoral immune responses to viral nucleocapsid

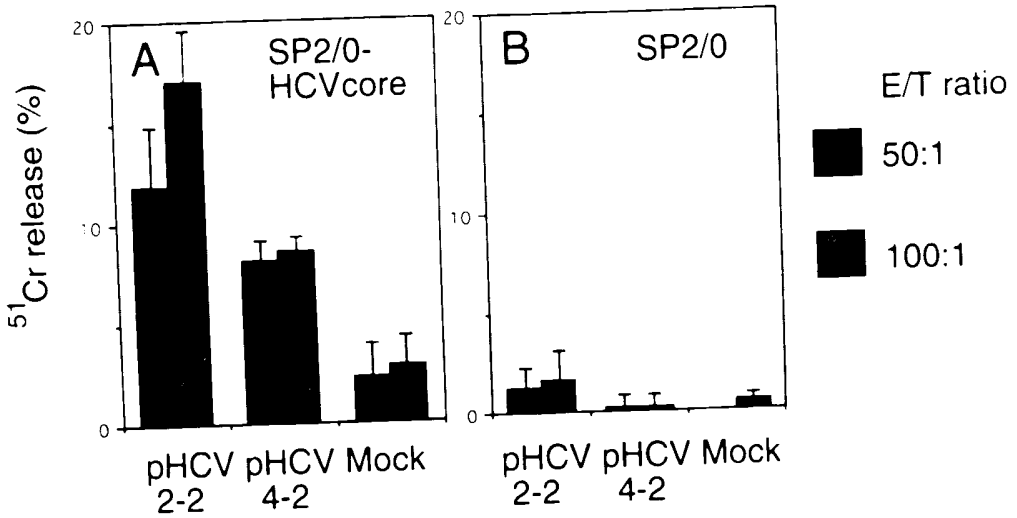

FIG. 7. Specific CTL activity against SP_2/O cells expressing (A) the HCV core antigen compared with (B) native SP_2/O cells. All animals received four immunizations with pHCV 2-2, pHCV 4-2, or mock DNA constructs prior to preparation of splenocyte suspensions.

proteins are the findings with the nucleoprotein of the lymphocytic choriomeningitis virus. Like HCV core, the nucleoprotein of lymphocytic choriomeningitis virus was not secreted when expressed in cells from a transfected plasmid DNA construct. Yokoyama et al.[55] and Martin et al.[46] have reported that a plasmid-based DNA vaccine construct comprised of lymphocytic choriomeningitis virus nucleoprotein induced weak or no humoral immunity in mice following injection into muscle cells, presumably because such cells neither express major histocompatibility complex class II antigens nor stimulate $CD4^+$ helper T and B cells by secretion from the cell. It is likely that only a small fraction of plasmid DNA is taken up and expressed by antigen-presenting cells residing in and around muscle tissue. Therefore, it is reasonable to postulate that the humoral immune response induced by HCV core DNA immunization was weak because of the fact that the core protein is not secreted from the cell to be processed by antigen-presenting cells.[56]

The recent study by Lagging et al.[54] also demonstrated that a HCV core DNA vaccine construct generated CTL activity against HCV core epitopes *in vitro*. However, in this study, it was necessary to use spleen cells derived from immunized mice that were previously stimulated by cells infected with vaccina virus encoding the HCV core gene to demonstrate such CTL activity. We found that the pHCV 2-2 construct generated substantial CTL activity in all mice immunized with this construct, because tumor cell growth was inhibited and the animal survival rate was improved. Thus, CTL activity was highly operative *in vivo* against tumor cells in this animal model system. Furthermore, spontaneous CTL activity was observed *in vitro* as well, because SP_2/O cells expressing HCV core epitope were specifically killed by splenocytes derived from HCV core–immunized mice. Regarding the comparison between pHCV 2-2 and 4-2 constructs, pHCV 2-2 was more effective in generating CTL activity than pHCV 4-2. It is reasonable to postulate that the large amount of core protein produced intracellularly by the pHCV 2-2 construct induced CTL more easily than the smaller amount produced by pHCV 4-2.

It was of interest to determine whether the HCV-DNA constructs would improve the survival rate of mice after tumor challenge. In the mock DNA–immunized normal mice, all died by 21 days after tumor injection (mean survival, 18 days). In contrast, all mice immunized with pHCV 2-2 survived over the observation period, suggesting that CTL activity was present in all pHCV 2-2–immunized animals. Previous studies have also demonstrated that DNA-based immunization may improve animal survival rate in a tumor model. For example, Wang et al.[44] reported that, in human immunodeficiency virus–DNA–immunized mice, at 16 weeks, 90% were completely protected against tumor formation induced by SP_2/O cells expressing the gp160 protein of human immunodeficiency virus. In our studies, the tumors derived from the pHCV 2-2–immunized mice were small and grew slowly, but eventually developed in all HCV core–vaccinated animals. One difference may possibly be caused by the tumor load. Wang et al. challenged mice with 3×10^6 SP_2/O cells, but, in the present investigation, we used 1×10^7 SP_2/O cells.

Our studies suggest that strong CTL activity may be generated to a nonsecreted HCV core protein both *in vitro* and *in vivo*, and that the pHCV 2-2 construct is a promising antiviral agent to test as a therapeutic agent during persistent HCV infection. Finally, polynucleotide vaccination with such a DNA construct may also have value as an immunization approach for prevention of acute HCV infection as well.

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